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ON THE GROUP SPECIFICNESS OF ANTIBODIES IN ANTISTREPTOCOCCUS SERUM

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In previous articles I[1] have shown that sheep immunized with hemolytic streptococci from scarlet fever and erysipelas, respectively, produced specific opsonins and agglutinins. The results of absorption tests also indicated that the streptococci from these diseases belonged to distinct biologic groups. Protection experiments with the streptococci from scarlet fever gave additional weight to this inference in respect to the scarlatinal group. Bliss[2] and Gordon[3] reached similar conclusions concerning streptococci from scarlet fever, by means of agglutination tests with immune rabbit serum.

TECHNICAL

In my experiments the animals were immunized first with heat-killed organisms and later with living. The cocci were grown in ascitic dextrose broth for 24 hours, centrifuged and suspended in salt solution and then heated at 60 C. for one hour. When living cocci were injected, they were grown in goat blood agar slants for 24 hours and the growth washed off in salt solution and injected intravenously. The killed organisms were first injected subcutaneously into rabbits and later intravenously. All of the injections into the sheep were intravenous. As a rule, the animals were inoculated every day for 4 days with increasing doses followed by a rest of 4 days. This was repeated 2 or 3 times, and then weekly doses were given for a month or more. The growth on 1 blood agar slant was the maximum dose of living organisms for rabbits, the growth on 3 for sheep. Larger doses stopped antibody production. The animals were generally bled 4 days after an injection.

For the opsonic experiments, the cocci were grown on goat blood agar for 24 hours and suspended in salt solution. Normal leukocytes, sheep with sheep serum and human with rabbit serum collected in 0.2%

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[1] Jour. Am. Med. Assn., 1920, 74, p. 1386, and 75, p. 1339.

[2] Bull. Johns Hopkins Hosp., 1920, 31, p. 173.

[3] Brit. Med. Jour., 1921, 1, p. 632.

sodium citrate solution and washed once in salt solution, were used. The serum, normal and immune, was heated for one-half hour at 56 C. to remove the thermolabile element, and then diluted with salt solution. The mixtures of diluted serum leukocytes and coccal suspensions, equal parts, were incubated for 25 minutes, smears stained with carbol thionin, 100 polymorphonuclear leukocytes counted, and the number of cells taking part in phagocytosis noted. The point of opsonic extinction was determined by finding the dilution at which opsonification ceased.

In the agglutination tests cocci were grown from 24 to 48 hours in ascitic dextrose broth, 1 part of ascitic fluid to 4 parts of 1 per cent. dextrose broth P_H 7.8. The dextrose broth was made with Fairchild's cultural peptone. The cultures were centrifuged, the supernatant fluid removed and the cocci suspended in salt solution. At first the serum dilutions were made with plain broth but later salt solution was used, the final dilutions running from 1:20 to 1:800, an equal part of bacterial suspension being added to each tube of diluted serum. Tubes containing cocci in broth or salt without serum and also mixtures of normal serum at the same dilutions as the immune serum were included in each test. The immunizing streptococcus was tested in each experiment to serve as a standard. The mixtures were incubated from 1 to 3 hours at 55 C. Stored serum sometimes agglutinated more slowly than others, but as a rule agglutination was complete at the end of 1 hour. Ascitic fluids of low specific gravity, say 1.008, with a P_H 7.8 are particularly suitable for this work. A specific gravity of 1,020 in one instance caused spontaneous clumping of all the strains of streptococci. Some lots of broth caused spontaneous clumping, the reason for which was not determined.

The absorption tests were made in different ways. The organisms were grown in ascitic dextrose broth for 24 hours, centrifuged, suspended in salt solution, heated at 60 C. for 1 hour, centrifuged and the serum to be absorbed added to this, in order to dilute the serum as little as possible. Care was taken that the absorbed serum was not diluted in higher dilutions than the unabsorbed. At first the suspended cocci and serum were incubated 2 hours at 37 C., refrigerated for 24 hours, and finally centrifuged, the supernatant serum being removed. This process was repeated 3 or 4 times. Later the mixtures were incubated over night at 37 C. and this repeated if the serum was still found to agglutinate the immunizing coccus. Incubation at 55 C. for varying periods was less successful.

SHEEP

The serum from the sheep immunized for 5 months with the streptococcus from scarlet fever, specifically agglutinated and opsonified hemolytic streptococci from scarlet fever, after 11 months' storage in the icebox. It was necessary, however, to activate the serum with fresh sheep serum (one part in 9 of immune serum) to demonstrate the opsonins.

This sheep, after 10 months' immunization, produced agglutinins and opsonins for hemolytic streptococci from sources other than scarlet fever. It was found difficult to absorb these nonspecific agglutinins. Absorption was repeated 4 times. Absorption with a streptococcus from erysipelas and 1 from acute tonsillitis failed to remove the agglutinins, except in one instance in which the tonsillitis streptococcus removed the agglutinins for its own and 2 scarlet fever streptococcus strains. On the other hand, the immunizing scarlet fever streptococcus in every case absorbed antibodies for the heterologous but rarely the homologous scarlet fever streptococcus, and never those from other sources. No explanation is offered for these results. The experiments were repeated several times.

Another sheep was now immunized as before with a hemolytic streptococcus from the throat of a patient with an acute case of scarlet fever. This streptococcus was agglutinated and opsonified in high dilutions by the stored immune sheep serum, specific for scarlet fever. After 3 weeks' immunization the serum agglutinated 4 strains of streptococci from scarlet fever, but not 5 strains from other sources. After being stored in the icebox for 2 days it lost its specificity, agglutinating nonscarlatinal strains in as high a dilution as scarlatinal. On absorption with the homologous coccus over night at 37 C., the agglutinins for 4 scarlet fever strains were absorbed, but not 4 from other sources, which would indicate that the serum was specific but contained minor agglutinins in large amounts. At the next bleeding, one week later, the serum specifically agglutinated streptococci from scarlet fever, but the following day it agglutinated some nonscarlatinal strains. The third bleeding, one week later, gave serum which agglutinated 8 strains of scarlet fever streptococci, but not 14 strains from other sources. This specimen lost most of its agglutinating power after two days in the icebox, but did not show any indication of nonspecificity. The serum from the next bleeding, one week later, agglutinated streptococci from all sources in the same dilution as the homologous scarlet fever strain 1 : 200. Four days later fresh serum agglutinated the homologous strain at a dilution of 1 : 4,000, one scarlet fever strain at 1 : 36,000, a nonscarlatinal strain at 1 : 20. The following days this serum agglutinated the immunizing strain at only 1 : 20. No agglutinins were demonstrable after the next injection, 8 weeks after the first. The serum was examined as soon as drawn, and after a few weeks standing in the icebox, to determine whether an inhibiting substance as described by Coca and Kelly[4] was present. None was demonstrated. Three days following the next inoculation agglutinins were present in dilutions as high as 1 : 2,400 for streptococci from scarlet fever, but not for those from other sources. This serum agglutinated the homologous streptococcus in a dilution of 1 : 80 only, and these only after 3 hours' incubation. This specimen of serum remained specific, but after 3 days in the icebox agglutinated no strain in a higher dilution than 1 : 20. No agglutinins could be demonstrated after the next inoculation.

A sheep immunized with a hemolytic streptococcus from erysipelas produced agglutinins and opsonins specific for streptococci from erysipelas for 4 months, then ceased to produce agglutinins, although opsonification was a marked property of the serum. No agglutinins were demonstrated for 4 months, then some were produced in a dilution of 1 : 50 for streptococci from erysipelas and also those from other sources. Absorption experiments were made with the homologous erysipelas streptococcus, a scarlet fever and tonsillitis streptococcus and a staphylococcus albus. The serum was absorbed over night at 37 C. for 2 successive nights as one absorption did not remove the agglutinins for the homologous coccus. The agglutinins were removed by all 3 strains of streptococci but not by the staphylococcus.

Another sheep was immunized for 2 months with a hemolytic streptococcus from tonsillitis. Neither agglutinins nor opsonins were produced for the immunizing coccus except occasionally agglutinins could be demonstrated at a dilution of 1 : 20. When agglutinins were present they were not specific, but were found only in very low dilutions.

Hooker and Anderson[5] call attention to the great importance of taking into consideration the individuality of the animal in studies of antibody specificity. They showed that only a few rabbits produced specific antibodies for human erythrocytes. In some of their experiments the specificity for iso-agglutinative corpuscular groups entirely disappeared after the second and third series of injections, but remained unchanged for over 4 months in the immune serum obtained from the first bleeding. In other rabbits the specificity was not impaired by subsequent injections.

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[4] Jour. Immunol., 1921, 6, p. 87.

[5] Jour. Immunol., 1921, 6, p. 419.

RABBITS

Hamilton and Havens,[6] Dochez, Avery and Lancefield,[7] Bliss [2] and Gordon [3] have produced specific hemolytic streptococcus agglutinins in rabbits. Dochez and his co-workers observed, however, that rabbits varied a good deal, some showing a wide range of cross agglutination and their serum was less specific than that of sheep. Evans [8] and Browning and Wilson,[9] found that apparently only certain rabbits produced antibodies.

First I immunized 6 rabbits with 2 strains of hemolytic streptococci from scarlet fever, 1 from tonsillitis and 1 from sinusitis. Agglutinins were readily produced, but no specificity could be demonstrated. Opsonins were present in low dilution and showed no specificity. The agglutinins were absorbed as thoroughly by one streptococcus as by another, streptococci from scarlet fever, erysipelas, sinusitis and tonsillitis being used. These results would indicate that common agglutinins only were produced in the rabbits immunized in this case.

Immunization with few and small doses: As it has been shown by various observers (Friedberger and Dorner,[10] Lake, Osborne and Wells,[11] Butterfield and Neill,[12] Hooker and Anderson [5]) that animals may develop a serum of higher specificity after a few small doses and that increased polyvalency develops after further injections, fewer and somewhat smaller doses were tried in 5 rabbits. Hemolytic streptococci isolated from the throats of patients in the early stages of scarlet fever were used for immunization. The cocci were grown in 10 c c of ascitic dextrose broth for 24 hours, centrifuged, suspended in salt solution and heated at 56 C. for 30 minutes. This suspension was either injected intravenously in 1 dose or in a few instances in 3 doses on the same day. After 2 weeks small numbers of living cocci (one eighth of the growth on a blood agar slant) were injected intravenously every week or two to keep up antibody production. One rabbit was injected with cocci killed by suspending them in a saturated glucose solution over night at 37 C., centrifuging and replacing the glucose solution with salt.

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[6] Jour. Am. Med. Assn., 1919, 72, p. 272.

[7] Jour. Exper. Med., 1919, 30, p. 179.

[8] U. S. Hyg. Lab. Bull., 1920, No. 124, p. 56.

[9] Jour. Immunol., 1920, 5, p. 417.

[10] Centralbl. f. Bakteriol., O, I., 1905, 38, p. 544.

[11] Jour. Infect. Dis., 1914, 14,, p. 364.

[12] U. S. Hyg. Lab. Bull., 1920, No. 124, p. 15.

Two rabbits produced no agglutinins. About one week after the first injection, the other rabbits showed specific agglutinins in their serum in dilutions from 1 : 160-1 : 1,280, but lost their agglutinating power in a few days on standing in the icebox. The heterologous scarlet fever streptococci were generally agglutinated in as high dilutions as the immunizing coccus. After receiving living organisms, the rabbits sometimes produced specific agglutinins in higher dilutions for varying periods, one as long as 4 months. Further injections have so far produced nonspecific serums. The serum of one rabbit remained specific for 3 months in the icebox. The serum of the rabbit immunized with glucose-killed streptococci agglutinated scarlatinal streptococci in higher dilutions (1 : 10,000) than the others. This may have been accidental but is in accord with experiments made by Dr. Weaver and myself,[13] which showed that greater antibody production and protection was obtained after immunization with galactose-killed streptococci than by heat-killed streptococci.

For the agglutination tests the streptococci were now grown in phosphate broth, the formula used by Havens and Taylor.[14] Meat infusion broth was made with distilled water, to which was added 1% peptone, 1% disodium phosphate and 0.5% glucose. It was adjusted to P_H 8-8.2. Tubes containing 10 c c of the broth were autoclaved at 10 pounds for 10 minutes. The organisms grew profusely in this medium, producing, as a rule, a perfectly homogeneous suspension. Occasionally it was necessary to transfer a culture several times in this broth in order to get rid of spontaneous clumping. The agglutination experiments were made as before except that the serum was diluted with phosphate broth and the cultures in phosphate broth used for the suspensions. The specimens were incubated for 1 hour at 55 C. Each experiment contained strains of the immunizing and another known scarlatinal streptococcus and 2 strains of known heterologous hemolytic streptococci as controls.

The absorption tests were made according to the method of Gordon,[3] the immune serum being saturated with the various strains of streptococci to be examined, the absorbed serums then being tested with the immunizing coccus, a strain which grew homogeneously and clumped readily with specific immune serum was selected for immunization. The organisms were grown in phosphate broth for 24 hours, centrifuged, the supernatant fluid removed and the immune serum, diluted 1 : 10 with broth, added to this very thick mass of cocci. As the different strains grew equally well, each tube contained approximately the same number of organisms. The tubes were incubated at 55 C. for 2 hours and centrifuged, and agglutination tests were made with the supernatant fluid. If the immunizing coccus did not absorb the agglutinins in this time, the speciments were kept in the icebox over night and the supernatant fluid added to more cocci as before. It was rarely necessary to repeat the absorption more than twice. Fresh serum appeared to absorb more readily than stored serum. Here again each experiment was controlled with phosphate broth without serum, normal serum, unabsorbed immune serum, and immune serum absorbed with the homologous and another known scarlatinal streptococci and 2 known heterologous hemolytic streptococci.

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[13] Jour. Infect. Dis., 1908, 5, p. 589.

[14] Am. J. Hygiene, 1921, 1, p. 311.

Seventy-five strains of hemolytic streptococci have been examined for agglutinins with specific immune rabbit serum. Thirty-nine of these strains, 22 scarlatinal and 17 heterologous, were used for absorption tests. Thirty-six cultures were isolated from the throats of patients in the early stages of scarlet fever or from patients with its complications: purulent rhinitis (3), otitis media (3), mastoiditis (1). cervical adenitis (2), lochia (1), wound (1), and burn (1). The discharge from the cervical abscess of one of the patients had persisted for 4 months. The 39 nonscarlatinal strains were taken from patients with acute tonsillitis among attendants in the Durand hospital, discharges from ear and cervical glands of diphtheria patients, otitis media, mastoiditis, smallpox pustule, paranephritic abscess, peritonsillar abscess and the infected finger of the nurse of a scarlet fever patient.

None of the cultures were agglutinated by normal rabbit serum.

All of the strains from the scarlet fever patients except 3 were agglutinated in high dilutions by the specific immune serum. One of these was a mannite fermenter. Only 3 strains fermenting mannite have been encountered in about 250 hemolytic streptococcus cultures; all were from scarlet fever and none were agglutinated by the immune serum. Another strain which was not agglutinated was isolated from the lochia of a puerperal sepsis patient with clinical scarlet fever. In this case no hemolytic streptococci were isolated from the throat at the onset of the infection. The third culture was isolated from a nurse with an acute sore throat, a faint general erythema, most marked around the elbows, followed by arthritis and desquamation on the hands and feet. None of these 3 strains absorbed the specific agglutinins from the immune serum.

In two other cases of surgical scarlet fever, hemolytic streptococci isolated from the wound on the forehead and from a burn on the arm, as well as from the throat, were agglutinated by the immune serum.

A hemolytic streptococcus isolated from the finger of a nurse with a sore throat was shown to belong to the scarlatinal group both by agglutination and absorption tests. The throat culture was not so agglutinated.

Two patients admitted with a diagnosis of diphtheria with profuse purulent secretion from the nose, one with enlarged cervical glands, which discharged later, showed no diphtheria bacilli, but almost pure cultures of hemolytic streptococci. Absorption and agglutination tests indicated their scarlatinal origin. A diphtheria patient in the same room contracted a typical attack of scarlet fever except for desquamation 4 days later, which suggested that the patients had had scarlet fever, although there was no such history.

A severe case of scarlet fever in a child, following a cleft palate operation, infected 3 nurses. One had a typical attack of scarlet fever, one a severe sore throat with little eruption, while the third showed only sore throat. The hemolytic streptococci from the patient and the three nurses were all agglutinated in high dilutions by the immune serum. The streptococcus from the nurse without any exanthem absorbed the agglutinins as readily as those from the patient and the nurse with a typical eruption. This case appears to be in the same class as 2 described by Bliss [2] and 2 by me [15] of hemolytic streptococci specific for scarlet fever from patients exposed to scarlet fever who had acute tonsillitis without exanthem.

The other nonscarlatinal strains were not agglutinated by the immune serum. Absorption tests with 17 indicated also that they did not belong to the scarlatinal group of streptococci.

The absorption experiments corresponded in every case to the agglutination tests.

SUMMARY

Immune sheep serum containing specific opsonins and agglutinins for hemolytic streptococci from scarlet fever retained its specific antibodies for eleven months in the icebox.

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[15] Jour. Infect. Dis., 1921, 29, p. 91.

The serums of 2 sheep, specific for hemolytic streptococci from scarlet fever and erysipelas, respectively, produced nonspecific streptococcus opsonins and agglutinins after 8 to 10 months' immunization.

During the course of immunization of a sheep with a hemolytic streptococcus from scarlet fever, some lots of serum became nonspecific on standing in the icebox; some were nonspecific when drawn; some were specific in dilutions above 1 : 20. Later the serum was specific for streptococci from scarlet fever and remained so on standing in the icebox, but it lost its agglutinating power rapidly.

Rabbits immunized with frequent large doses of hemolytic streptococci from scarlet fever, tonsillitis and sinusitis, produced only nonspecific agglutinins and opsonins. Rabbits immunized with infrequent and somewhat smaller doses of hemolytic streptococci from scarlet fever generally produced specific agglutinins.

Some sheep and rabbits do not produce antibodies in any appreciable amount.

Agglutination and absorption experiments with specific immune rabbit serum confirm previous observations that most of the hemolytic streptococci associated with scarlet fever belong to a distinct biologic group.